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Structure and expression of several bean (*Phaseolus vulgaris*) nuclear transfer RNA genes: relevance to the process of tRNA import into plant mitochondria

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Abstract

Bean nuclear genes for $tRNA^{Pro}$, $tRNA^{Thr}$and $tRNA^{Leu}$ were isolated. Expression of the $tRNA^{Pro}$ genes was demonstrated *in vivo* and sequence analysis suggested amplification of the $tRNA^{Pro}$ gene copy number through duplication of a gene cluster at the same locus of the bean genome. The two $tRNA^{Thr}$ genes isolated were actively transcribed and their transcripts processed in a HeLa cell system. *In vivo* expression tests of these genes and aminoacylation assays of the corresponding *in vitro* transcripts showed the presence of identity determinants in the anticodon of plant $tRNA^{Thr}$. The $tRNA^{Leu}$ gene was not expressed due to deviation from the consensus in the internal B-box promoter. The same sequence deviation also prevented aminoacylation of the corresponding *in vitro* transcript. This $tRNA^{Leu}$ however exists in plants and is synthesized from another gene with a consensus B-box promoter. Plant mitochondria import from the cytosol a number of nucleus-encoded tRNAs, including $tRNA^{Leu}$ and $tRNA^{Thr}$. From the available sequence data, we could not identify any conserved structural motif characteristic for the nucleus-encoded tRNAs imported into plant mitochondria, either in the tRNAs, or in the gene flanking sequences. These results suggest that recognition of tRNAs for import is idiosyncratic and likely to depend on protein/RNA interactions that are specific to each tRNA or each isoacceptor group.

Introduction

Information about plant nuclear transfer RNA (tRNA) genes is growing slowly, as compared to animals and yeast, and genes for a number of tRNA species are still unknown. Some of the identified genes have been shown to exist as families which are dispersed throughout the plant genome, like *Nicotiana rustica* $tRNA^{Tyr}$ and $tRNA^{Ser}$ genes [1, 2] or *Arabidopsis thaliana* $tRNA^{Trp}$ genes [3]. Several tRNA genes may also be 'clustered' at a single chromosomal site as are 4 $tRNA^{Pro}$ genes and 2 pseudogenes in the bean nuclear genome [4]. A cluster of 2 $tRNA^{Tyr}$ genes and a $tRNA^{Ser}$ gene is present in at least 20 copies at a single chromosomal site in the genome of *A. thaliana* [5].

In animals and fungi, transcription of nuclear tRNA genes primarily depends on internal control regions (the 'A' and 'B' 'boxes') within the tRNA gene (reviewed in [6]) corresponding to the 5′ part of the DHU stem and loop (A-box, TRGYNNARY GG, positions 8–19 in the classical tRNA nucleotide numbering [7]; position 17 is absent for some tRNAs and is not included in the consensus) and to the TΨC loop (B-box, GT/ATCNANNC, positions 53–61). Additional promoter elements in the 5′ and in some cases in the 3′-flanking regions contribute to transcription regulation (reviewed in [8]). The A- and B-box internal promoters are conserved in plant tRNA genes and are essential for transcription of the rice $tRNA^{Gly}$ gene in a

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X98179, X98183, X98184 and Y15080.

yeast *in vitro* assay, although sequences in the 5′ flanking region may also modulate the expression of this gene [9]. *In vivo* plant systems based on the translational suppression of stop codons in reporter genes in the presence of suppressor variants of the tRNA genes to be tested have been successfully developed [10, 11] and have permitted the analysis of the role of 5′ and 3′ flanking sequences in the expression of plant nuclear $tRNA^{Trp}$ genes [3] and of a plant nuclear $tRNA^{Leu}$ gene [12]. *In vitro* expression of plant nuclear tRNA genes has only been assayed in heterologous animal or yeast systems, apart from one study concerning transcription termination with wheat RNA polymerase III [13].

Several new plant nuclear tRNA genes were isolated in the course of the work presented here. Investigating their structure and expression led to interesting observations concerning the requirement for the internal control units in plant nuclear tRNA genes, amplification of plant nuclear tRNA genes, and plant tRNA aminoacylation and identity elements.

Previous studies established the complex genetic origin of higher-plant mitochondrial tRNAs (e.g. [14]). There is a clear evolutionary tendency towards loss from the plant mitochondrial DNA of the 'native' tRNA genes inherited from the ancestral endosymbiotic genome (e.g. [15]). A number of tRNAs are therefore no longer encoded by the mitochondrial genome but are imported from the cytosol [16]. As a consequence, part of the nuclearly encoded tRNAs partition between the cytosol and the mitochondria [17]. The mechanism of mitochondrial tRNA import in plants is still unknown. Among other possibilities, one can speculate that some cytosolic tRNAs are transported into plant mitochondria due to special properties in their sequence or in the structure of their precursor [14]. Extended 'precursor' forms of some cytosolic tRNAs have been detected in the mitochondria of trypanosomatids which are totally deprived of mitochondrial tRNA genes and import all their tRNAs from the cytosol [18]. In this context, we analyzed the structure of different plant nuclear genes coding for mitochondrially imported tRNAs or tRNAs present only in the cytosol with the aim of searching for conserved characteristic structural motifs, including in the flanking regions.

Materials and methods

Genomic cloning

Clones containing tRNA genes were isolated by screening a bean genomic library in the lambda EMBL-4 vector [19] with bean total cytoplasmic tRNA labelled with tRNA nucleotidyltransferase [20]. The plant DNA was recovered from the positive recombinant phage and suitable subclones retaining hybridization to the cytoplasmic tRNA probe were prepared in the plasmid Bluescript KS(+) (pKS, Stratagene) according to standard protocols [21]. Inserts were sequenced by the dideoxyribonucleotide chain termination method using a simplified protocol [22].

Isolation of bean total cytoplasmic and mitochondrial tRNAs and northern analysis

Total cytoplasmic tRNA was extracted from etiolated bean hypocotyls by phenol extraction and recovered by ethanol precipitation [20]. Large RNAs were eliminated by selective precipitation in 1 M NaCl and tRNAs were further purified by DEAE-cellulose chromatography [23]. The proportion of mitochondrial tRNAs in such preparations usually does not exceed 0.5% [23]. Total mitochondrial tRNA was prepared following similar methods [20] from bean mitochondria purified according to Neuburger *et al.* [24]. Cytoplasmic and mitochondrial tRNAs were fractionated by electrophoresis on 15% (w/v) polyacrylamide gels under denaturing conditions [25] and blotted onto membranes as described previously [17]; tRNA blots were probed with T4 polynucleotide kinase-labelled oligonucleotides (oligonucleotides 1, 2 and 3 for $tRNA^{Pro}$, $tRNA^{Thr}$ and $tRNA^{Leu}$, respectively).

Transient expression of tRNA genes in plant protoplasts

For expression in plant protoplasts, a CTA anticodon capable of reading amber stop codons was introduced into the tRNA genes by oligonucleotide-directed mutagenesis [26] using oligonucleotides 4, 5 and 6 for $tRNA^{Pro}$, $tRNA^{Thr}$ and $tRNA^{Leu}$, respectively. The resulting clones were verified by sequencing [22].

The $tRNA^{Pro}$amber suppressor derivatives were electroporated into potato protoplasts together with a plasmid, p35SGUS/Pro, derived from p35SGUS/WT [27]. p35SGUS/WT produces a translational fusion between the N-terminus of the polyprotein of the

tobacco Tnt1 retrotransposon and β-glucuronidase (GUS). To obtain p35SGUS/Pro, the proline codon in the Tnt1 sequence was switched to an amber stop codon by site-directed mutagenesis with oligonucleotide 7. Translation of these constructs in a plant transient expression system goes first through the Tnt1 sequence and therefore suppression of the amber codon is required to express GUS activity from p35SGUS/Pro. The amber suppressor derivatives of $tRNA^{Thr}$ and $tRNA^{Leu}$ were electroporated into tobacco or potato protoplasts together with the plasmid GUS/AMBER or p35SGUS/AMBER [27] which carry an amber codon instead of the second leucine codon in the GUS gene. The extra T at position 55a in the $tRNA^{Leu}$(AAG) gene was deleted by site-directed mutagenesis using oligonucleotide 8. A plasmid containing the luciferase marker gene [28] was co-electroporated with the tRNA and GUS constructs, and luciferase activity was used as a control. Construction of the GUS plasmids, preparation of protoplasts, electroporation conditions, assays for GUS and luciferase activities have been described previously [11, 27, 29].

Transcription of tRNA genes in a HeLa cell in vitro *system*

In vitro transcription of the bean nuclear tRNA genes using a HeLa cell nuclear extract was done as recommended by the manufacturer (Promega). Following incubation, the reaction medium was diluted with 7 volumes of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1% (w/v) SDS and extracted with water-saturated phenol. After ethanol precipitation, transcripts were analysed on a 15% polyacrylamide gel in denaturing conditions [25].

T7 RNA polymerase in vitro *transcription and aminoacylation of tRNA transcripts*

The plasmids containing the cloned tRNA genes were used as templates for PCR amplification with the relevant primers (oligonucleotides 9 and 10 for $tRNA^{Thr}$, 11 and 12 for $tRNA^{Leu}$) to obtain constructs in which the tRNA gene sequence was directly fused to the T7 RNA polymerase promoter at the 5′ terminus and to a *Bst*NI site (CCAGG) at the 3′ terminus. PCR products were cloned into the *Eco*RI site of pUC18 (Boehringer) following standard protocols [21] and sequenced [22]. *In vitro* run-off transcription of these constructs with T7 RNA polymerase after digestion with *Bst*NI yielded full-length tRNA transcripts with a 3′ CCA end. Transcripts were prepared according to Perret *et al.* [30]. Aminoacylation was conducted under optimal conditions [31] in the presence of saturating amounts of a bean hypocotyl cytoplasmic enzymatic extract [20] and of *L*-[^{3}H]leucine or *L*-[^{3}H]threonine (Amersham).

Reverse transcription and PCR amplification (RT-PCR and PCR)

For $tRNA^{Leu}$ cDNA synthesis, total cytoplasmic tRNA (5 μg) was treated three times with RNase free DNase under conditions recommended by the manufacturer (Pharmacia) and used for reverse transcription with oligonucleotide 14 [32]. One fifth of the reaction was taken for PCR amplification with oligonucleotides 13 and 14. For direct amplification of $tRNA^{Leu}$ genes, total bean nuclear DNA (1 μg) was used in a PCR reaction with either oligonucleotides 13 and 14 or oligonucleotides 15 and 16. For amplification of the junctions between the initially isolated *Rsa*I sub-fragments of the $tRNA^{Pro}$ gene cluster, the isolated 8.5 kb *Eco*RI bean genomic fragment (0.1 ng) was used in a PCR reaction with either oligonucleotides 17 and 18, 19 and 20, or 21 and 22. PCR products were cloned into the *Eco*RI site of pKS and sequenced [22].

Southern hybridization

Bean genomic DNA (7 μg per sample) was digested with several restriction endonucleases, fractionated on a 0.7% (w/v) agarose gel and transferred onto a nylon membrane (Hybond-N+, Amersham) in 0.4 M NaOH. Blots were hybridized with a random priming-labelled probe [33] synthesized from the $tRNA^{Thr}$ sequence. Hybridization was at 65 °C in 6× SSC, 0.5% (w/v) SDS [21]. Membranes were washed at the hybridization temperature twice in 2× SSC and once in 2× SSC, 0.1% SDS.

Oligonucleotides (all 5′→3′)

For northern hybridization:
(1) GGGGCATTCCGAGAATCGAAC ($tRNA^{Pro}$)
(2) ACCTTCAGTTTACAAGACTGACGCTC ($tRNA^{Thr}$)
(3) GACCAACTCGGCCATCTCAAC ($tRNA^{Leu}$)

For constructing amber suppressor tRNA genes (anticodon position underlined):
(4) GATTCTCGCTT<u>CTA</u>GTGCGAGAGGTC ($tRNA^{Pro}$)
(5) ACCTTCAGTTT<u>TAG</u>AGACTGACGCTC ($tRNA^{Thr}$)
(6) GCGCCAGATT<u>CTA</u>GTTCTGGTCCG ($tRNA^{Leu}$)

For mutating a proline codon in p35SGUS/WT and yield the p35SGUS/Pro construct:
(7) CTGACCTACCCGCTAATCTCTCATCCTTC (amber codon underlined)
For deleting T55a in the $tRNA^{Leu}$ gene:
(8) AGGGCGTGGGTTCAAATCCC (position of the mutation underlined)
For PCR amplification of T7 promoter/*Bst*NI constructs (*Eco*RI sites for cloning in italics):
(9) AGCAA*GAATTC*GAATTGTAATACGACTCACTATAGCCCTTATAGCTCAGTGGTAGAG ($tRNA^{Thr}$; T7promoter sequence underlined)
(10) GTACA*GAATTC*CCTGGTGCCCTCACGCAGGATCGAACTA ($tRNA^{Thr}$; *Bst*NI site underlined)
(11) AGCAA*GAATTC*GAATTGTAATACGACTCACTATAGTTGAGATGGCCGAGTTGGTCTAAG ($tRNA^{Leu}$; T7 promoter sequence underlined)
(12) GTACA*GAATTC*CCTGGTGTTGAGAGTGGGATTTGAA ($tRNA^{Leu}$; *Bst*NI site underlined)
For RT-PCR or PCR amplification of $tRNA^{Leu}$ (*Eco*RI sites for cloning in italics):
(13) AGCAA*GAATTC*GTTGAGATGGCCGAG (5′ end)
(14) GTACA*GAATTC*TGGTGTTGAGAGTGGG (3′ end)
(15) AGCAA*GAATTC*TGTGGAAAATTCTCATGA (5′ flanking)
(16) GTACA*GAATTC* AGATTAAAAATAAAAAAAG AC (3′ flanking)
For PCR amplification of the junctions between the initially isolated *Rsa*I sub-fragments of the $tRNA^{Pro}$ gene cluster (*Eco*RI sites for cloning in italics):
(17) TGGTT*GAATTC*CTTAAACCTTCAATGCTCCA C
(18) GTACA*GAATTC*GTTTTATGTATAAACTTTCTC CG
(19) AGGTT*GAATTC*GCTATCTTGAGCCTGCTTGC
(20) GTACA*GAATTC*CATGAAATCAGTAAGTTGGT TTC
(21) AGCAA*GAATTC*CTGTATTAGCACTACTTGCA C
(22) GATCT*GAATTC*CGACAATGACTTCAGACACA C

Results

Isolation of bean nuclear tRNA genes

Screening of a bean genomic library using total cytoplasmic tRNA yielded several positive clones, some of which were also recognized by a total mitochondrial tRNA probe. Restriction analysis, subcloning and sequencing led to the isolation of two $tRNA^{Pro}$(TGG) genes (*trn*P1(TGG) and *trn*P2(TGG) on *Rsa*I DNA fragments of 1431 bp and 524 bp, respectively, included in accession Y15080), a $tRNA^{Pro}$(AGG) gene (*trn*P(AGG) on a 1105 bp *Rsa*I DNA fragment included in accession number Y15080), two $tRNA^{Thr}$(TGT) genes (*trn*T1 on a 907 bp *Rsa*I DNA fragment, X98183, and *trn*T2 on a 421 bp *Dra*I DNA fragment, X98184, both derived from the same 5 kb *Eco*RI sub-fragment of a genomic clone) and a $tRNA^{Leu}$(AAG) gene (on a 419 bp *Alu*I DNA fragment, X98179).

The two $tRNA^{Pro}$(TGG) genes are identical to each other and to the previously isolated bean $tRNA^{Pro}$(TGG) genes, whereas the $tRNA^{Pro}$(AGG) gene is identical to the previously sequenced bean $tRNA^{Pro}$(AGG) gene [19].

The two $tRNA^{Thr}$ genes are the first ones described for higher plants. They code for the same tRNA species with only a single difference at position 48 (T or C) (Figure 1A) but their respective flanking sequences are very different. Nucleotide 48 is a pyrimidine which, in the tRNA three-dimensional structure, interacts with a purine at position 15, thus forming the 'Levitt-pair' [34]. This R_{15}-Y_{48} pair is normally a classical Watson-Crick pair, which in this case holds true for only one of the sequences.

When folding the sequence of the isolated $tRNA^{Leu}$ gene into the cloverleaf structure, an extra T appears in the TΨC loop (position '55a') between the consensus Ψ_{55} and C_{56} (standard tRNA nucleotide numbering [7]) (Figure 1B). The presence of an extra nucleotide at position 55a introduces a deviation from the consensus sequence into the conserved B-box internal promoter for tRNA gene transcription by RNA polymerase III in animal cells, in fungi and presumably in plants (e.g. [8, 35]) and therefore makes the expression of this $tRNA^{Leu}$ gene questionable.

Expression of the $tRNA^{Pro}$(TGG) genes

Northern analysis of total bean cytoplasmic and mitochondrial tRNA using a probe (oligonucleotide 1) specific for the $tRNA^{Pro}$(TGG) genes and, with a single

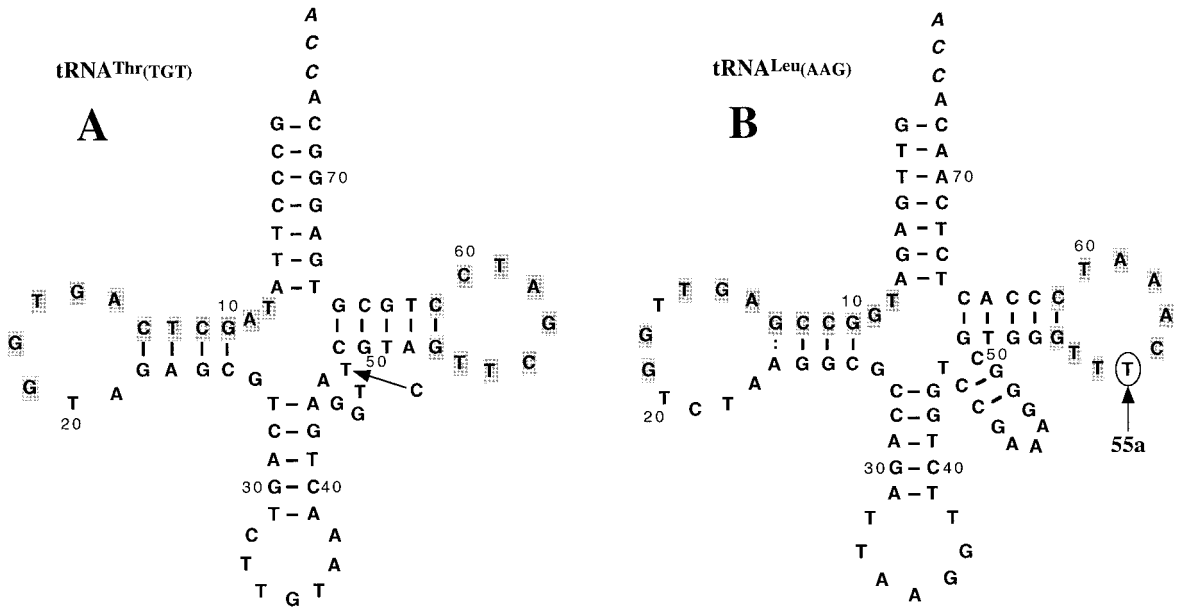

Figure 1. Cloverleaf folding of the sequence of the bean nuclear tRNA^{Thr}(TGT) (A) and tRNA^{Leu}(AAG) (B) genes. The C/T difference at position 48 between the two tRNAs^{Thr}, as well as the extra T nucleotide at position 55a in the tRNA^{Leu}, are indicated by arrows. The putative internal promoters (box A in the DHU stem and loop, box B in the TΨC loop) are highlighted with a shaded background.

nucleotide mismatch, for the tRNA^{Pro}(AGG) gene, yielded as expected a hybridization signal only with the cytoplasmic tRNA (Figure 2A). Proline tRNAs are not mitochondrially imported in the angiosperms studied so far [15]. This result also confirms the absence of significant cytosolic contamination in our mitochondrial tRNA fractions. Expression of the tRNA^{Pro}(TGG) genes was further tested in an *in vivo* translational suppression assay [10, 11]. After mutation of the anticodon to CTA, the *trn*P1 gene with 64 bp upstream and 719 bp downstream sequence, and the *trn*P2 gene with 278 bp upstream and 166 bp downstream sequence were assayed in potato protoplasts for suppression of an amber stop codon in a GUS gene construct. Measurement of GUS activity (Figure 2B) demonstrated that both tRNA^{Pro} genes were functional.

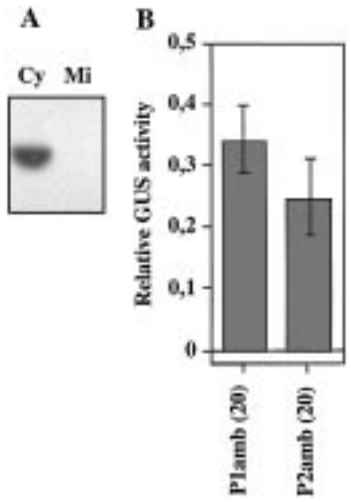

Figure 2. Expression studies of bean tRNA^{Pro}(TGG). A. Northern blot hybridization of an oligonucleotide probe specific for bean tRNA^{Pro}(TGG) to total bean cytoplasmic (Cy) and mitochondrial (Mi) tRNA. B. *In vivo* amber suppressor activity of bean tRNA^{Pro}; potato protoplasts were co-electroporated with the p35SGUS/Pro plasmid carrying an amber codon in the GUS gene construct and an amber suppressor gene derived from bean *trn*P1 (P1amb) or *trn*P2 (P2amb); 20 μg of the plasmid carrying the tRNA gene were applied; GUS activity was calculated relative to the activity obtained with the same batch of protoplasts after electroporation of the p35SGUS/WT plasmid which does not have the amber codon; relative activities are the means of two experiments; error bars indicate the standard error.

Expression of the tRNA^{Thr}(TGT) genes

Northern analysis of total bean cytoplasmic and mitochondrial tRNA using a probe (oligonucleotide 2) specific for the tRNA^{Thr}(TGT) genes demonstrated the existence of this tRNA *in vivo* and its presence in both subcellular compartments (Figure 3A). Threonine tRNAs are mitochondrially imported in all higher plants studied so far [15]. Amber derivatives of the *trn*T1 gene with 79 bp upstream and 138 bp down-

stream sequence, and the *trn*T2 gene with 181 bp upstream and 166 bp downstream sequence were assayed as suppressors in tobacco protoplasts using a GUS reporter gene with a premature amber stop codon. Neither of the two tRNA^{Thr} genes led to GUS activity (not shown), suggesting that either the genes or their transcripts were not functional.

The wild-type and amber suppressor tRNA^{Thr} genes were amplified by PCR from the corresponding clones using oligonucleotides 9 and 10 to yield constructs suitable for *in vitro* transcription with T7 RNA polymerase. The obtained mature-sized tRNA^{Thr} transcripts were aminoacylated in the presence of threonine and a partially purified bean enzymatic extract [20]. The wild-type tRNA^{Thr} transcripts were fully active, reaching 100% charging, whereas no significant aminoacylation with threonine could ever be detected with the amber suppressor tRNA^{Thr} transcripts (Figure 3B).

The conclusion of both series of experiments was that, as in the case of *Escherichia coli* [36] and yeast [37], the anticodon of the plant tRNA^{Thr} contains important identity determinants towards threonyl-tRNA synthetase which are lost when switching from (UGU) to (CUA). Expression of both original tRNA^{Thr} genes with natural upstream and downstream sequences, as well as of the corresponding amber derivatives, was finally obtained in the presence of a HeLa cell nuclear extract and the transcripts were processed to a mature tRNA size in the assays (Figure 3C). This implies that the tRNA^{Thr}(TGT) genes we have isolated are functional.

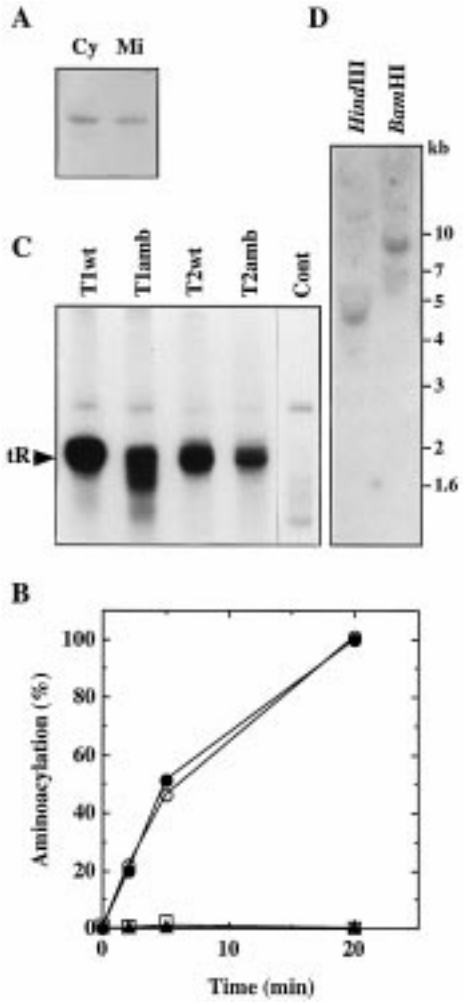

Figure 3. Expression and functional studies of bean tRNA^{Thr}(TGT). A. Northern blot hybridization of an oligonucleotide probe specific for bean tRNA^{Thr}(TGT) to total bean cytoplasmic (Cy) and mitochondrial (Mi) tRNA. B. Aminoacylation of bean tRNA^{Thr}(TGT) *in vitro* transcripts (4 μM) with threonine in the presence of a saturating amount of a partially enriched bean cytoplasmic enzymatic extract; the symbols correspond to the wild-type transcripts derived from *trn*T1 (○) and *trn*T2 (●) or to the amber derivatives from *trn*T1 (□) and *trn*T2 (▲). C. *In vitro* transcription of bean tRNA^{Thr}(TGT) genes in the presence of a HeLa cell nuclear extract; lanes correspond to the wild-type forms of *trn*T1 (T1wt) and *trn*T2 (T2wt) or to the amber derivatives of *trn*T1 (T1amb) and *trn*T2 (T2amb); a control was run without added DNA (Cont); migration of the mature size tRNA^{Thr}(TGT) transcript on the same gel is indicated (tR). D. Southern blot hybridization of a tRNA^{Thr}(TGT) random priming-labelled probe to bean genomic DNA (7 μg per lane) digested with *Hin*dIII or *Bam*HI.

Expression of the tRNA^{Leu}(AAG) gene

Northern analysis of total bean cytoplasmic and mitochondrial tRNA using a probe (oligonucleotide 3) specific for the tRNA^{Leu}(AAG) gene showed the existence of the corresponding tRNA *in vivo* and its import into mitochondria (Figure 4A). However, the amber derivative of this tRNA^{Leu} gene with 115 bp upstream and 222 bp downstream sequence was not active in a translational suppression assay (Figure 4B), whereas suppression was observed following deletion of the extra T55a (Figure 4B).

Constructs for T7 RNA polymerase *in vitro* synthesis of mature-sized tRNA^{Leu}(AAG) variants were amplified by PCR from the corresponding clones using oligonucleotides 11 and 12. Aminoacylation tests of these transcripts in the presence of leucine and purified bean leucyl-tRNA synthetase [38] showed that, whereas both the wild-type and the amber tRNA^{Leu} were inactive, deletion of the T55a allowed efficient aminoacylation (Figure 4C). Similarly, when the ori-

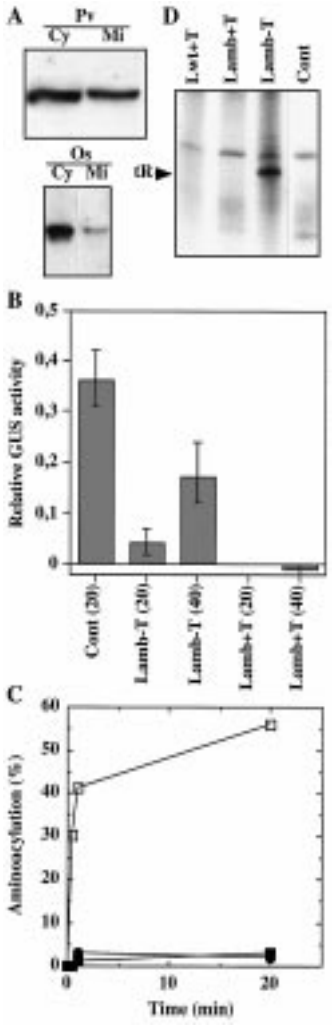

Figure 4. Expression and functional studies of bean tRNA^{Leu}(AAG). A. Northern blot hybridization of an oligonucleotide probe specific for bean tRNA^{Leu}(AAG) to total bean (Pv) or rice (Os) cytoplasmic (Cy) or mitochondrial (Mi) tRNA. B. *In vivo* amber suppressor activity of bean tRNAs^{Leu}; tobacco protoplasts were co-electroporated with the p35SGUS/AMBER plasmid containing a premature amber stop codon in the GUS gene, a luciferase control gene and a gene encoding an amber derivative of bean tRNA^{Leu}(C∗AA) (Cont) [25], or of bean tRNA^{Leu}(AAG) with (Lamb+T) or without (Lamb-T) the extra T at position 55a; 20 or 40 μg of the plasmid carrying the tRNA gene were applied; GUS activity was calculated relative to the control luciferase activity and corresponds to the means of three experiments; error bars indicate the standard error. C. Aminoacylation of bean tRNA^{Leu}(AAG) *in vitro* transcripts (4 μM) with leucine in the presence of a saturating amount of purified bean cytosolic leucyl-tRNA synthetase; the symbols correspond to the transcript synthesized from the originally isolated gene (●), the amber derivative (■) and the amber derivative without the extra T at position 55a (□). D. *In vitro* transcription of bean tRNA^{Leu}(AAG) genes in the presence of a HeLa cell nuclear extract; lanes correspond to the originally isolated gene (Lwt+T), the amber derivative (Lamb+T) and the amber derivative without the extra T at position 55a (Lamb-T); a control was run without added DNA (Cont); migration of the mature size tRNA^{Leu}(AAG) transcript on the same gel is indicated (tR).

ginal constructs with the natural 115 bp upstream and 222 bp downstream sequences were transcribed in the presence of a HeLa cell nuclear extract, expression was restricted to the T55a-deleted tRNA^{Leu} gene (Figure 4D). Thus, the presence of the extra T at position 55a apparently blocks both transcription of the tRNA^{Leu} gene and aminoacylation of the tRNA.

In a further set of experiments, RT-PCR was used with oligonucleotides 13 and 14 as primers to amplify the putative tRNA^{Leu}(AAG) from total bean tRNA. Amplification products were cloned and the 16 cDNAs analyzed all showed the sequence of the tRNA^{Leu}(AAG) without the extra T in the TΨC loop, definitely establishing the existence of this tRNA but implying that it originates from a different gene than the one which had been isolated. This second copy of the tRNA^{Leu}(AAG) gene could be amplified by classical PCR with the same oligonucleotides 13 and 14 as primers and bean genomic DNA as template. Surprisingly, the 30 independent PCR clones sequenced in this case were all deprived of the extra T55a. Whether this reflected a high copy number of the RNA^{Leu}(AAG) gene without T55a could not be reliably established with Southern blots of bean genomic DNA because probing with a specific oligonucleotide gave too weak signals and using a random priming-labelled probe led to uninterpretable results due to sequence similarities with other tRNA^{Leu} genes (not shown). Nevertheless, that the initially isolated bean tRNA^{Leu} gene was not a cloning artifact was demonstrated by PCR amplification using bean genomic DNA as template and as primers oligonucleotides 15 and 16 corresponding to the immediate 5′- and 3′-flanking sequences of this gene. In this case, all 24 PCR clones analyzed had the tRNA^{Leu}(AAG) sequence with the extra T55a.

We conclude that tRNA^{Leu}(AAG) is encoded by at least two genes in the bean genome and that we have initially isolated a gene copy which is inactive, very likely because of an insertional mutation in the B-box internal promoter.

A computer search enabled us to identify a so far undetected tRNA^{Leu}(AAG) gene 391 nucleotides upstream of an anther-specific gene (RTS2) in a rice (*Oryza sativa*) genomic DNA sequence (J.Y.K. Lee and T.K. Hodges; GenBank accession number U12171). The rice tRNA^{Leu} gene is 100% identical to the bean tRNA^{Leu}(AAG) gene described here without the T55a. The corresponding tRNA is present in rice total cytoplasmic and mitochondrial tRNA fractions, as shown by northern hybridization (Figure 4A). The absence of significant cytosolic contamination

in the rice mitochondrial tRNA fraction was confirmed using the $tRNA^{Pro}$ probe (oligonucleotide 1) (not shown). Using sunflower RNA as a template and primers derived from the 5′ and 3′ ends of the previously described bean $tRNA^{Leu}$(C∗AA) [25], Ceci *et al.* [39] amplified by RT-PCR a cDNA corresponding to a $tRNA^{Leu}$(AAG). The specifically amplified sequence (N_{25}–N_{48}) is 100% identical to the corresponding region in the bean $tRNA^{Leu}$(AAG) presented in this work. The $tRNA^{Leu}$(AAG) we have characterized is therefore likely to be generally expressed in both dicotyledonous and monocotyledonous plants.

Structural analysis of plant nuclear tRNA genes and tRNAs. Search for conserved features

The 8.5 kb *Eco*RI fragment from which the $tRNA^{Pro}$ genes studied in this work were isolated was actually derived from the same λEMBL4 bean genomic clone as the 6.5 kb *Eco*RI fragment previously shown to bear a $tRNA^{Pro}$ gene cluster [4]. Sequence alignment established extensive conservation of the flanking regions between each $tRNA^{Pro}$ gene from one *Eco*RI fragment and a corresponding $tRNA^{Pro}$ gene from the other *Eco*RI fragment (Figure 5). PCR amplification of the intervening sequences between the initially isolated *Rsa*I subclones of the 8.5 kb fragment allowed us to establish a continuous sequence of about 4.5 kb (accession number Y15080) and showed that the order of the $tRNA^{Pro}$ genes was also conserved between the 6.5 and 8.5 kb *Eco*RI genomic fragments (Figure 5). The *trn*P(AGG), *trn*P1(TGG) and *trn*P2(TGG) on the 8.5 kb fragment share about 160/400, 690/230 and 760/170 nucleotides of upstream/downstream sequence with the $tRNA^{Pro}$(AGG), $tRNA^{Pro}(UGG)_2$ and $tRNA^{Pro}(UGG)_3$ (as they were called previously [4]) on the 6.5 kb fragment. A further 170 nucleotide element (SR) is conserved upstream of the two $tRNA^{Pro}$(AGG) genes (Figure 5) and shorter homologous motifs (30–90 nucleotides) were found scattered in the two fragments. Altogether, these sequence homologies strongly suggest that a duplication of an initial unit containing a $tRNA^{Pro}$(AGG) and two $tRNA^{Pro}$(TGG) genes occurred in the bean $tRNA^{Pro}$ gene cluster. Finally, whereas the 6.5 kb fragment was shown previously to contain an additional $tRNA^{Pro}$(TGG) gene to this unit, as well as two $tRNA^{Pro}$ pseudogenes, hybridization studies demonstrated that no further $tRNA^{Pro}$ gene and no gene for another tRNA was present in the remaining unsequenced part of the 8.5 kb fragment.

Isolation of the first higher-plant nuclear $tRNA^{Thr}$ genes allowed us to align sequences corresponding to the three nucleus-encoded tRNA species which are clearly imported from the cytosol into the mitochondria in all higher plants tested so far, namely $tRNA^{Ala}$, $tRNA^{Leu}$ and $tRNA^{Thr}$ [15, 40]. An *A. thaliana* $tRNA^{Ala}$ gene sequence has been published previously [41] and this gene was subsequently amplified by PCR from rapeseed (*Brassica napus*) and potato (*Solanum tuberosum*) [31]. Mitochondrial import of the *A. thaliana* $tRNA^{Ala}$ was directly proven in tobacco transgenic plants [42]. Bean $tRNA^{Leu}$(C∗AA) was the first plant tRNA which was shown to be shared by the cytosol and the mitochondria [25] and its mitochondrial import was demonstrated in transgenic potato plants [17]. Both the $tRNA^{Ala}$ and the $tRNA^{Leu}$ gene are present as single copies or at least at low copy number [25, 41], which makes it very likely that they are a source for the tRNAs to be mitochondrially imported and validates comparisons of flanking sequences. Southern blot hybridization of bean genomic DNA showed that the $tRNA^{Thr}$ genes described in this work are also likely to be present as single copies or at low copy number (Figure 3D), considering that both genes were isolated from the same 5 kb *Eco*RI bean genomic fragment. Mitochondrial import of $tRNA^{Thr}$ has been implied in a number of plant species [15, 16] and confirmed by the above northern hybridizations (Figure 3A).

Ignoring the anticodons and the invariant and semi-invariant nucleotides, alignment of the $tRNA^{Ala}$, $tRNA^{Leu}$ and $tRNA^{Thr}$ sequences revealed a few conserved structural features (e.g. C_{25}, G_{26}, a G_{30}:C_{40} base-pair) (Figure 6A). Only some of these features remained conserved when considering also $tRNA^{Arg}$ (mitochondrially imported in all angiosperms tested but not defined yet in gymnosperms [15]) and $tRNA^{Val}$ (probably also imported in all higher plants, although its situation is not definitely clear in potato [40]), as well as genes for $tRNA^{Ala}$, $tRNA^{Arg}$, $tRNA^{Leu}$, $tRNA^{Thr}$ and $tRNA^{Val}$ whose sequences were recently obtained from systematic sequencing programs and whose expression is not yet documented (Figure 6B). On the other hand, none of these conserved features turned out to be specific to imported tRNAs when compared to sequences of nuclearly encoded $tRNA^{Asp}$, $tRNA^{Gln}$ and $tRNA^{Tyr}$ which have never been found to be imported into plant mitochondria [15] (Figure 6C). Comparing the flanking regions of the $tRNA^{Ala}$, $tRNA^{Leu}$ and $tRNA^{Thr}$ genes did not reveal significant sequences which would be specific-

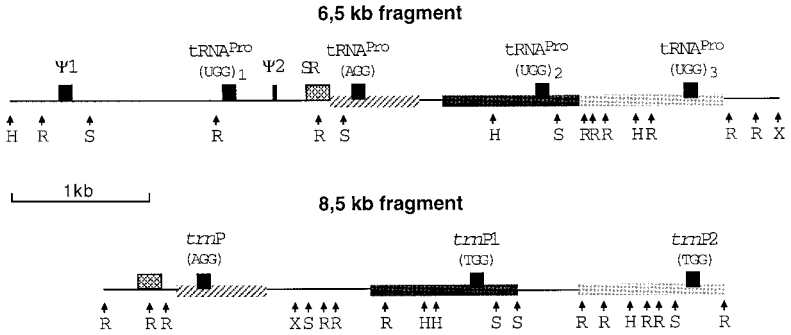

Figure 5. Sequence homologies in the tRNA^{Pro} gene clusters between the 8.5 kb *Eco*RI bean genomic fragment studied in this work and the previously analyzed 6.5 kb *Eco*RI fragment of the same genomic clone [4]. Filled boxes represent tRNA^{Pro} genes (tRNA^{Pro}, *trn*P) and pseudogenes (Ψ1 and Ψ2); long highly homologous regions (>85%) in the flanking sequences of the tRNA^{Pro} genes are hatched or dotted; a 170 nucleotide sequence (SR) upstream of the tRNA^{Pro}(AGG) in the 6.5 kb fragment was also found conserved in the 8.5 kb fragment. Finally, fine comparison between the two tRNA^{Pro} gene clusters revealed a pattern of shorter homologous motifs (30–90 nucleotides) which is not given in detail in the figure. Sites for *Hin*dIII (H), *Rsa*I (R), *Sph*I (S) and *Xba*I (X) restriction endonucleases are indicated by arrows.

ally conserved upstream (or downstream) of these nuclear genes coding for tRNAs to be targeted to mitochondria (Figure 7A and C). Also, no peculiar feature was observed upon computer analysis of potential secondary structure folding of gene flanking sequences. The analyses were extended to the flanking sequences of the recently made available tRNA^{Ala}, tRNA^{Arg}, tRNA^{Leu}, tRNA^{Thr} and tRNA^{Val}genes (Figure 7B and D). Altogether, there is no indication so far that there exist characteristic features in the precursors to mitochondrially imported tRNAs.

Discussion

The present work describes the first nucleus-encoded tRNA^{Thr} genes in higher plants (tRNA^{Thr} nuclear genes have also been described recently for the lower plant *Marchantia polymorpha* [43]) and complements the data available for other plant nuclear tRNA gene families, with a novel tRNA^{Leu} gene sequence and information on a possible tRNA^{Pro} gene duplication. Our results further illustrate the diverse organization of tRNA genes in the plant nuclear genome. The tRNA^{Pro} gene cluster previously identified [4] turned out to be even larger than originally proposed and to be made up of at least 7 functional genes plus two pseudogenes. On the contrary, the non functional copy of the tRNA^{Leu}(AAG) gene that we have isolated is the only tRNA gene present in the corresponding genomic clone and it is not clustered with the active copy that we amplified by PCR. Finally, the tRNA^{Thr} gene is organized in two copies that are clustered in a single fragment of a genomic clone. Nine potentially functional tRNA^{Pro} genes are present within a region of less than 7 kb in the *A. thaliana* genome (Rounsley *et al.*, *A. thaliana* BAC clone T01B08, accession number U78721), indicating that clustering of tRNA^{Pro} genes might be conserved among plant species. Analysis of the expression of the bean tRNA^{Leu}(AAG) genes isolated in this work argues for the use of the B-box internal promoter in plant nuclear tRNA genes, as the gene copy with an extra nucleotide in this box was not transcribed in a HeLa cell *in vitro* system and no evidence was found for *in vivo* expression of this copy in protoplasts or in plants. Similarly, it was shown previously that an *A. thaliana* tRNA^{Ser}(AGA) gene with a non-consensus nucleotide in the B-box (a T at position 56 instead of the invariant C) is not transcribed *in vitro* [44].

Sequence alignment and folding did not reveal any structural motif which is conserved and characteristic for the nuclearly encoded tRNAs imported into plant mitochondria, either in the tRNAs themselves, or in the gene flanking sequences. The latter makes it unlikely that precursors of tRNAs to be targeted into mitochondria share a specific 5′ extension deriving from the upstream region of the genes. Imported tRNAs carrying long 5′ extensions have been observed in *Trypanosoma brucei* mitochondria [18], but sequencing established that these 5′ extensions did not derive from the upstream regions of the corresponding nuclear tRNA genes [45]. Moreover, subsequent work has shown that the sequences flanking tRNA genes encoding imported tRNAs in trypanosomatids have no role in the import process [46, 47]. Thus, the origin of the 5′ exten-

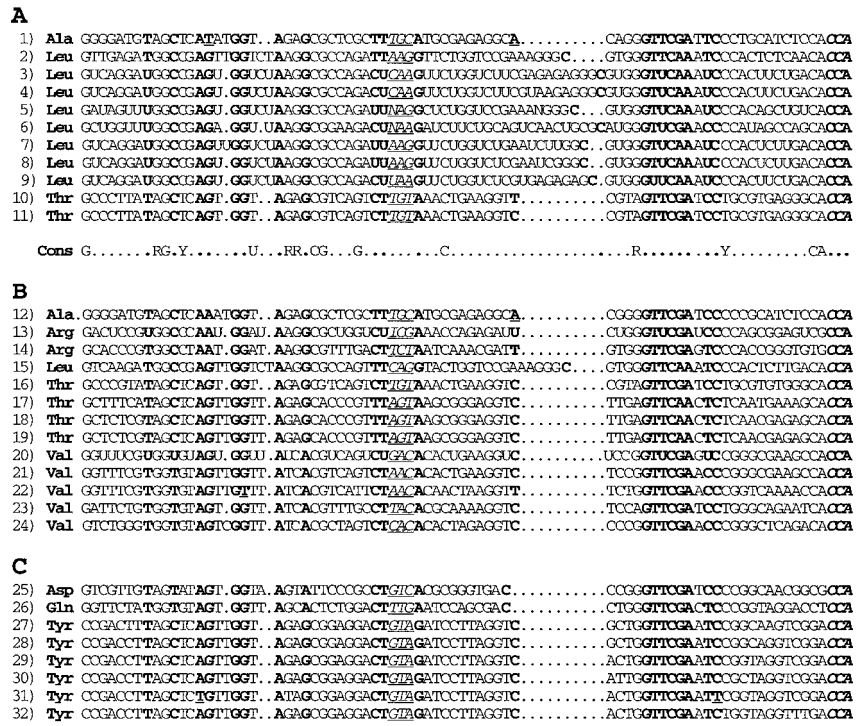

Figure 6. Sequence alignment of mitochondrially imported (A and B) or non-imported (C) tRNAs in plants. 1, *A. thaliana* $tRNA^{Ala}$(TGC) [41]; 2, bean $tRNA^{Leu}$(AAG) (this work); 3, bean $tRNA^{Leu}$(C*AA) [25]; 4, potato $tRNA^{Leu}$(CAA) [49]; 5, bean $tRNA^{Leu}$(NAG) [50]; 6, bean $tRNA^{Leu}$(NAA) [51]; 7 and 8, lupin $tRNAs^{Leu}$(AAG) [52]; 9, lupin $tRNA^{Leu}$(UAA) [52]; 10 and 11, bean $tRNA^{Thr}$(TGT) (this work); 12, *A. thaliana* $tRNA^{Ala}$(TGC) (*A. thaliana* chromosome 1; BAC F7G19, accession AC000106); 13, wheat $tRNA^{Arg}$(ICG) [53]; 14, *A. thaliana* $tRNA^{Arg}$(TCT) (*A. thaliana* chromosome 5, P1 clone, MED24, accession AB005235); 15, *A. thaliana* $tRNA^{Leu}$(CAG) (*A. thaliana* chromosome 4 ESSA I contig, fragment 6, ATFCA6, accession Z97341); 16, *A. thaliana* $tRNA^{Thr}$(TGT) (*A. thaliana* chromosome 2, BAC T01O24, accession AC002335); 17, *A. thaliana* $tRNA^{Thr}$(AGT) (*A. thaliana* chromosome 4, ESSA I contig, fragment 6, ATFCA6, accession Z97341); 18, *A. thaliana* $tRNA^{Thr}$(AGT) (*A. thaliana* chromosome 4, ESSA I contig, fragment 1, ATFCA1, accession Z97336); 19, *A. thaliana* $tRNA^{Thr}$(AGT) (*A. thaliana* chromosome 4, ESSA I contig, fragment 5, ATFCA5, accession Z97340); 20, lupin $tRNA^{Val}$(GAC) [54], 21) *A. thaliana* $tRNA^{Val}$(AAC) [55], 22) *A. thaliana* $tRNA^{Val}$(AAC) (*A. thaliana* BAC IG002N01, accession AF007269); 23, *A. thaliana* $tRNA^{Val}$(TAC) (*A. thaliana* chromosome 4, ESSA I contig, fragment 5, ATFCA5, accession Z97340); 24, *A. thaliana* $tRNA^{Val}$(CAC) (*A. thaliana* chromosome 5, P1 clone, MXA21, accession AB005247); 25, soybean $tRNA^{Asp}$(GTC) [56]; 26, *A. thaliana* $tRNA^{Gln}$(TTG) [57]; 27, wheat $tRNA^{Tyr}$(GTA) [58]; 28 and 29, *Nicotiana rustica* $tRNAs^{Tyr}$(GTA) [1]; 30 to 32, *A. thaliana* $tRNAs^{Tyr}$(GTA) [5]. Data are from direct tRNA sequencing (3–9, 13, 20) or from sequencing of nuclear tRNA genes (1, 2, 10–12, 14–19, 21–32). Anticodons are underlined and in italics; invariant and semi-invariant nucleotides are in bold; deviations from invariant and semi-invariant nucleotides are both underlined and in bold; in the case of genes, CCA ends, which are not encoded, are in italics. Conserved nucleotides (Cons) refer to the set of mitochondrially imported alanine, leucine and threonine tRNAs presented in A; invariant and semi-invariant positions were not considered for comparison. Nucleotides conserved in alignment A are not all conserved in alignment B. None of the nucleotides conserved in the imported tRNAs is characteristic when aligned with the sequences of non-imported tRNAs C.

sions and the relevance of these extended tRNAs to the import process remain to be clarified.

The results presented here, together with the varied pattern of tRNAs imported in different plants [15, 40], refute the hypothesis that mitochondrially imported tRNAs share characteristic structural features. It appears more likely that recognition of these tRNAs for import is due to idiosyncratic features of each tRNA or isoacceptor group. This in turn implies that multiple 'receptors' or 'carriers' might exist. The recent finding that a single base mutation in $tRNA^{Ala}$ that blocks recognition by plant alanyl-tRNA synthetase prevents mitochondrial import of this tRNA in transgenic plants [42], suggests that at least in some cases specific recognition of tRNAs for import might involve cognate aminoacyl-tRNA synthetases, as in yeast [48].

Figure 7. Sequence alignment of upstream (A and B) or downstream (C and D) flanking regions of nuclear genes encoding mitochondrially imported tRNAs. Numbering and references are as in Figure 6 except for 33: rice $tRNA^{Leu}$(AAG) (Lee and Hodges, accession U12171).

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